Heterogeneity of T-Cell Clones Infiltrating Primary Malignant Melanomas

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It is established that primary malignant melanomas (pMM) can be infiltrated by T-cell populations with predominantly one T-cell clone. As pMM generally express multiple tumor-associated antigens (TAA), here we used laser-capture microdissection (LCM) to isolate different tumor-infiltrating lymphocyte (TIL) clusters in order to determine whether pMM are infiltrated only by one single clone or whether the TAA may attract various T-cell populations. As T-cell receptor (TCR) clonality is a useful tool for the demonstration of specific T-cell clones, we analyzed 56 pMM, three cutaneous melanoma metastases, and 15 pairs of pMM with a sentinel lymph node (SLN) for clonal rearrangements of the (TCR) γ chain gene. We detected the clonality of TCR γ chain gene in 25 of 56 pMM, and in 10 of 17 SLN studied. In four of the 15 pairs of primary tumor and SLN, we found clonal TCR γ in both the melanoma and the SLN, with two pairs harboring the identical clone. As we detected different clones in pMM and the corresponding SLN, we subsequently performed LCM in 21 malignant melanomas with multiple lymphocytic clusters for the presence of focal clonal T cells in different regions of the melanoma. In seven melanomas, both clusters of TILs showed the same rearranged TCR γ chain gene and in five of the seven biopsies the clonal rearrangement occurred in different variable (V) regions of the TCR γ chain gene. These tumors showed infiltration by more than one clone. In 10 biopsies TCR clonality was restricted to one cluster, while the second microdissected sample of the infiltrate was polyclonal. In conclusion, within one primary malignant melanoma several T-cell clones with different rearrangements may occur. The balance between these clones may decide on the progress of melanoma.

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INTRODUCTION

Primary malignant melanomas usually harbor various amounts of tumor-infiltrating-lymphocytes (TIL). Tumor infiltration by TIL reflects a specific host response that results from recognition of tumor-associated antigens either on antigen-presenting cells or on the surface of the tumor cells by T lymphocytes (Van der Bruggen *et al.*, 2002).

The biological significance of the local T-cell-mediated immune response is difficult to evaluate, as in malignant melanoma the coexistence of either regression or progressive and invasive tumor margins is frequently associated with a dense lymphocytic infiltrate. Spontaneous regression is defined as the partial or complete disappearance of tumor masses without treatment (Cole, 1976) and is often histologically characterized by a dense lymphocytic infiltrate of T-helper cells (Lowes *et al.*, 1997).

Previous reports revealed that tumor-infiltrating lymphocytes (TIL) in malignant melanoma are frequently of clonal origin in both progressive and regressive parts of the identical tumor (thor Straten *et al.*, 1996) and in its metastases (thor Straten *et al.*, 1999). These findings raise the question of whether one T-cell clone may exert both tumor-promoting and tumor-inhibitory properties or whether tumors may present several antigens and attract different T-cell populations. Two very recent studies found in the peripheral blood and in melanoma metastases of patients vaccinated with MAGE antigens several different T-cell clones recognizing both the vaccinated antigen and other melanoma-associated antigens (Germeau *et al.*, 2005; Lurquin *et al.*, 2005). However, the clonal response against the primary malignant melanoma, and not the secondary metastasis without vaccination, remains unanswered.

Recent therapeutic trials in patients with metastatic melanoma use *in vitro* highly tumor-specific T-cell clones for adoptive transfer (Yee *et al.*, 2000) with variable success. As most of the vaccines used are major histocompatibility complex (MHC) I restricted, the MHC expression of the tumor was analyzed by performing immunohistochemistry with antibodies against MHC I and MHC II. We further characterized the TIL by staining for CD4 and CD8 to determine the

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Abbreviations: LCM, laser-capture microdissection; MHC, major histocompatibility complex; pMM, primary malignant melanoma; SLN, sentinel lymph node; TAA, tumor-associated antigen; TCR, T-cell receptor; TIL, tumor-infiltrating lymphocyte

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amount of cytotoxic CD8-positive lymphocytes needing MHC I expression for tumor detection or MHC-II-dependent CD4-positive T-helper cells.

Moreover, in malignant melanomas, antigen presentation occurs either by direct recognition of the tumor cells by cytotoxic T lymphocytes or by cross-presentation of tumor-antigens via antigen-presenting cells, which can activate both CD8- as well as CD4-positive TIL.

Determination of T-cell receptor (TCR) clonality is a tool for the characterization of cutaneous lymphocytic infiltrates (Holm *et al.*, 2002). A new technical approach for the detection of clonality is the usage of laser-capture micro-dissection (LCM). This technique allows isolating small clusters of cutaneous lymphocytic infiltrates. In a previous study using LCM to evaluate clonality in cutaneous T-cell lymphoma, we could demonstrate a superior sensitivity with equal specificity when combined with analysis of TCR γ chain gene rearrangement of the entire block (Yazdi *et al.*, 2003). This superior sensitivity of the LCM method allows us to detect several smaller clones instead of one dominant clone found by conventional clonality analysis.

In the present study, we used LCM to characterize various parts of the peritumoral infiltrate for TCR γ chain gene rearrangement in a large number of primary malignant melanomas and sentinel lymph node (SLN) biopsies. In sections showing a large amount of TIL forming distinct clusters, we separately analyzed microdissected lymphocytic infiltrates from several parts of the identical tumor for their TCR γ chain gene rearrangements and found that one single melanoma can be infiltrated by more than one T-cell clone.

RESULTS

TCR γ rearrangement in DNA extracted from total tumor sections

DNA extracted from paraffin blocks of a total of 56 primary malignant melanomas and three melanoma metastases was analyzed for rearrangement of the TCR γ chain gene. A clonal TCR γ chain gene rearrangement could be detected in 25 of 56 (45%) malignant melanomas and in one of three (33%) melanoma metastases screened (Table 1).

In 15 patients, we analyzed the primary tumor and the corresponding sentinel node. In four of the 15 patients, we found TCR γ chain gene rearrangement in both the melanoma and the lymph node. In order to assure clonality, we sequenced DNA in two of the four pairs of lymph nodes and primary malignant melanomas. The data confirmed that the clones were identical. One single T-cell clone was found in two of the 15 primary melanomas, while the other melanomas showed either multiple clones or polyclonal infiltrates. In contrast, four of the 17 SLNs harbored a clearly predominant T-cell clone (Table 2).

LCM reveals TCR heterogeneity at various sites of the infiltrate

As we found different T-cell clones in primary malignant melanomas and their corresponding SLNs, and as melanomas express more than one tumor-associated antigen (TAA), we speculated that it is unlikely that TIL are composed by only one single T-cell clone. We therefore performed LCM of two

Table 1. TCR γ gene rearrangement examining the entire lesion (conventional DNA extraction)

TNM	Clark	Breslow	Clonality
Tis	MM *in situ*	Polyclonal	
Tis	MM *in situ*	Polyclonal	
T1	III	0.44 mm	Vγ2
T1	II	0.2 mm	Vγ2; Vγ9; Vγ11
T1	III	0.6 mm	Vγ9
T1	III	0.5 mm	Vγ2
T1	II	0.3 mm	Vγ2; Vγ11
T1	II	0.3 mm	Vγ9; Vγ10
T1	III	0.32 mm	Vγ9
T1	III	0.25 mm	Vγ2; Vγ9
T1	II	0.24 mm	Polyclonal
T1	II	0.5 mm	Polyclonal
T1	III	0.42 mm	Polyclonal
T1	III	0.56 mm	Polyclonal
T1	III	0.4 mm	Polyclonal
T1	III	0.56 mm	Polyclonal
T1	III	0.36 mm	Polyclonal
T1	III	0.36 mm	Polyclonal
T1	III	0.4 mm	Polyclonal
T1	III	0.5 mm	Polyclonal
T1	III	0.5 mm	Polyclonal
T1	II	0.6 mm	Vγ2
T1	II	0.18 mm	Polyclonal
T1	III	0.23 mm	Polyclonal
T2	III	1.3 mm	Vγ2
T2	IV	1.4 mm	Polyclonal
T2	III	0.8 mm	Polyclonal
T2	III	1.3 mm	Polyclonal
T2	III	0.85 mm	Vγ2
T2	III	1.2 mm	Polyclonal
T3	III	1.8 mm	Vγ11
T3	IV	2.7 mm	Polyclonal
T3	III	1.8 mm	Vγ11;Vγ9
T3	III	1.8 mm	Vγ9
T3	IV	1.74 mm	Vγ11
T3	IV	2.1 mm	Vγ2; Vγ9
T3	IV	1.6 mm	Vγ2; Vγ11
T3	III	3.7 mm	Vγ11
T3	III	3.7 mm	Polyclonal
T4	IV	4.0 mm	Polyclonal
	MM metastasis		Vγ11
	MM metastasis		Polyclonal
	MM metastasis		Polyclonal

Table 2. T-cell receptor γ clonality of primary malignant melanoma with sentinel lymph node

TNM	Diagnosis	Clonality
T1	III; 0.6 mm	Vγ9
	LN w/o MM	Polyclonal
T3	III; 1.8 mm	Vγ9
	LN w/o MM	Polyclonal
T3	III; 1.5 mm	Polyclonal
	LN w/o MM	Vγ9
T3	III; 1.7 mm	Vγ9; Vγ11
	LN w/o MM	Vγ9.
T3	IV; 1.8 mm	Vγ9
	LN w/o MM	Vγ10
T3	III; 1.7 mm	Vγ10
	LN w/o MM	Vγ2
T2	III; 1.4 mm	Polyclonal
	LN w/o MM	Vγ2; Vγ11
T2	III–IV; 1.3 mm	Vγ9; Vγ10
	LN w/o MM	Vγ2
	LN w/o MM	Vγ2; Vγ9
T4	IV; 5.6 mm	Polyclonal
	LN with MM	Vγ2
	LN w/o MM	Vγ11
T2	III; 1.1 mm	Polyclonal
	LN w/o MM	Vγ2
T2	III; 1.0 mm	Polyclonal
	LN with MM	Polyclonal
T2	III; 1.2 mm	Polyclonal
	LN with MM	Polyclonal
T2	IV; 1.4 mm	Polyclonal
	LN w/o MM	Polyclonal
T3	IV; 1.6 mm	Polyclonal
	LN w/o MM	Polyclonal
T1	III; 0.54 mm	Polyclonal
	LN w/o MM	Polyclonal

LN with MM: sentinel lymph node with melanoma cells; LN w/o MM: sentinel lymph node without melanoma cells.

sites of the peri- or intratumoral infiltrate and compared TCR γ chain gene rearrangement in 21 primary malignant melanomas. These melanomas showed histologically at least two distant areas of patchy lymphocytic infiltrates of approximately 500–2,000 lymphocytes each. We found TCR clonality in at least one site in 17 of the infiltrates. In two out of the 17 infiltrates, two LCM sites revealed identical TCR γ chain gene rearrangement, while in 15 of the 17 infiltrates TCR γ chain gene rearrangement was different between the two LCM sites. Five of the 17 infiltrates showed in each site a different clone and the other 10 had a monoclonal infiltrate at one site and a predominantly polyclonal infiltrate at the other site under the conditions studied, meaning that no T-cell population exceeded 1% of the entire TIL population.

As cytotoxic (CD8+) lymphocytes either need antigen-presenting cells or HLA class I antigen expression, the HLA status of the melanomas with dense lymphocytic infiltrates was examined. In malignant melanoma, the antigen presentation can occur either by direct recognition of the tumor cells by cytotoxic T lymphocytes or by crosspresentation of tumor antigens via antigen-presenting cells, which can activate both CD8- as well as CD4-positive TIL. Therefore, additionally, the CD4/CD8 ratio of the TIL was determined using immunohistochemistry. As shown in Table 3, no correlation could be drawn between HLA expression and the amount of CD4- or CD8-labelled T lymphocytes and TCR γ gene rearrangement.

DISCUSSION

Tumors, especially melanomas, can evoke immune responses that include the activation of TAA-specific T cells (Romero *et al.*, 1998). TAA-specific T cells have been shown to interfere even with the growth of established tumors in the mouse (Greenberg, 1991; Egeter *et al.*, 2000) and in humans (Yee *et al.*, 2000; Dudley *et al.*, 2002). On the other side, tumor-specific TIL do not exclusively exert protective responses. Under still poorly defined conditions, tumor-specific immune responses may also facilitate tumor growth. Both regression and progression occur frequently simultaneously within one single melanoma. In order to elucidate the underlying mechanisms, we started by analyzing the heterogeneity of TIL within the lymphocytic infiltrate surrounding primary melanomas. As functional heterogeneity is difficult to determine in terms of DNA analysis, here we looked for differences in TCR γ chain gene rearrangement at different sites of the infiltrate using LCM. In agreement with published data, we found TCR γ chain gene rearrangements in 45% of primary malignant melanomas and in 59% of the tumor-draining SLNs. Interestingly, we could detect clonality in SLNs, the place where immune responses are triggered. In order to assure clonality, we sequenced DNA in two of the four pairs of lymph nodes and primary malignant melanomas. The data confirmed that the clones were identical. One explanation for this fact might be that a dominant T-cell clone is attracted by antigen-expressing melanoma cells or antigen-presenting cells and therefore showing a local expansion of a dominant clone.

The distribution of the TCR γ chain gene rearrangements at distinct areas could previously not be performed with DNA extracted from paraffin blocks. In studies published until now, the samples always resulted from the entire block. Thus, no statement can be given on locally expanded clones or the distribution of clonal T-cell lymphocytes. To determine whether TCR rearrangement is a focal event, we used an LCM-assisted approach. Aggregates of TIL from two different regions of one primary malignant melanoma were microdissected and separately analyzed for TCR γ chain gene rearrangement.

Table 3. Results of the rearrangement studies from two different microdissected clusters of tumor-infiltrating lymphocytes (LCM 1 and LCM 2) compared to the entire lesion and HLA expression of the lesion (HLA-A2 expression was graded by a homogenous expression (>75%), a heterogenous expression (25–75%) or negative expression of the melanoma cells)

TNM	Clark	Breslow	Entire lesion	LCM 1	LCM 2	CD4/CD8	HLA-A2	HLA-DP/DQ/DR
Tis		MM *in situ*	Polyclonal	Polyclonal	Polyclonal	CD8>CD4	<25%	Negative
Tis		MM *in situ*	Polyclonal	Vγ10	Polyclonal	CD4>CD8	75–100%	Negative
T1	III	0.6 mm	Vγ9	Vγ9	Polyclonal	CD8>CD4	25–75%	Positive
T1	III	0.46 mm	Vγ2	Vγ9	Polyclonal	CD4>CD8	<25%	Negative
T1	III	0.7 mm	Polyclonal	Vγ11	Vγ2	CD4=CD8	25–75%	Negative
T1	III	0.62 mm	Vγ10	Vγ2	Vγ10	CD4=CD8	75–100%	Negative
T1	III	0.3 mm	Polyclonal	Polyclonal	Polyclonal	CD4=CD8	<25%	Negative
T1	III	0.25 mm	Vγ2, Vγ9	Vγ10	Polyclonal	CD4=CD8	25–75%	Negative
T2	III	0.85 mm	Vγ2	Vγ2	Vγ9	CD4=CD8	75–100%	Positive
T2	IV	0.8 mm	Vγ2	Vγ9	Vγ9	CD4=CD8	75–100%	Negative
T3	IV	1.74 mm	Vγ11	Vγ2	Vγ9	CD8>CD4	<25%	Negative
T3	IV	3.3 mm	Vγ2, Vγ11	Vγ10	Vγ10, Vγ11	CD4>CD8	<25%	Positive
T3	IV	2.1 mm	Vγ2, Vγ9	Vγ11	Vγ2, Vγ9	CD4=CD8	75–100%	Negative
T3	III	1.7 mm	Vγ9, Vγ11	Polyclonal	Vγ10	CD8>CD4	25–75%	Negative
T3	IV	1.8 mm	Vγ9	Polyclonal	Polyclonal	CD4=CD8	75–100%	Negative
T3	III	1.7 mm	Vγ10	Polyclonal	Vγ2	CD8>CD4	25–75%	Negative
T3	III	1.8 mm	Vγ9	Polyclonal	Vγ9	CD4=CD8	<25%	Negative
T3	III	1.8 mm	Vγ9, Vγ11	Vγ11	Polyclonal	NA	NA	NA
T3	IV	1.6 mm	Vγ2, Vγ11	Polyclonal	Polyclonal	CD8>CD4	25–75%	Negative
T3	IV	2.2 mm	Polyclonal	Polyclonal	Vγ11	CD4=CD8	<25%	Positive
T3	IV	2.9 mm	Polyclonal	Vγ11	Polyclonal	NA	NA	NA

NA, no melanoma left on the paraffin block.

Comparing the two lymphocyte clusters, we detected a rearrangement of the identical variable (V) region in only two of the 21 tumors screened, with one tumor showing two different clones in one of the microdissected TIL. It is speculated that melanomas that harbor one single T-cell clone have a favorable outcome and a tendency for spontaneous regression (Ferradini *et al.*, 1993). Yet, other groups detected clonality in both regressive and progressive regions of primary malignant melanomas (Salvi *et al.*, 1995; thor Straten *et al.*, 1996). Histologically, there was no regression at any site and no substitution of tumor nests by fibrous tissue.

Five melanomas had clonal TCR γ chain gene rearrangements in two TIL clusters, but with various V regions rearranged, suggesting that TIL recognize more than one TAA in most tumors. These results are in line with a casuistic report by thor Straten *et al.* (1999), who found high numbers of locally expanded clonotypes in malignant melanoma metastases from two patients. Yet, thor Straten *et al.* (1999) used antibodies against specific TCR Vβ, a clustering of various Vβ families, in a metastasis. Even though this analysis does not allow to study clonal rearrangement, it suggests enrichment of various clones in melanoma metastasis.

Surprisingly, half of the melanomas (10 of 21) showed clonality in one TIL and a polyclonal infiltrate in the other. At the first glance, this focal distribution of a clonal T-cell subset depends neither on the thickness of the tumor nor on the presence of regression, as the TIL clusters were not microdissected from regressive regions.

Comparing the new LCM approach with clonality analysis of the entire section (Table 3), 45% of the melanomas screened carry one dominant clone detectable by conventional TCR γ chain rearrangement studies, while the new method enables us to detect additional focal clones. Interestingly, often the dominant clone was not found on LCM, as the dominant clone in some tumors does not have to be localized in the microdissected patchy infiltrates, but is rather situated in the sparse infiltrate localized in the upper papillary dermis.

In summary, our data show that TCR γ chain gene rearrangements are common in malignant melanoma. A clonal gene rearrangement could be detected in 45% of the 56 melanomas screened. The presence of clonal TIL is a focal event in most of the tumors analyzed, as we found different clones in various regions of the same primary malignant melanoma using LCM. Our results shed new light on the

paradox of large lymphocytic infiltrates in progressive and regressive areas of one single malignant melanoma (thor Straten *et al.*, 2000). As we previously showed that different T-cell populations have different effects on tumor growth (Egeter *et al.*, 2000; Mocikat *et al.*, 2003), the data here suggest that different types of TIL may either inhibit or promote melanoma growth in different areas of the same tumor.

MATERIALS AND METHODS

Study cases and design

A total of 95 formalin-fixed and paraffin-embedded biopsies were included in the study (Table 1). Samples included 56 primary cutaneous malignant melanomas and three melanoma metastases. In all, 15 pairs of a primary malignant melanoma with a SLN were screened. Altogether, 14 lymph nodes did not contain any tumor cells and three lymph nodes showed malignant melanoma cells (two melanomas had two SLNs excised) (Table 2). Samples were collected from the Dermatohistopathology Section of the Department of Dermatology, Ludwig-Maximilians-University Munich, and diagnosed by two dermatopathologists. Diagnoses were made according to standard dermatohistological practice.

Immunohistochemistry

Expression of CD4, CD8, MHC I (HLA-A2), and MHC II (HLA-DP, DQ, DR) was determined by immunohistochemistry. Briefly, 4-μm sections were placed on coated glass slides, pretreated in 1 mM EDTA (HLA-A2, CD4) or 10 mM citrate buffer (CD8) at 100°C for 5 minutes and incubated with monoclonal antibodies CD4 (Novocastra, UK), CD8 (Dako, Denmark), 0397HA and 0791HA (One Lambda, Canoga Park, CA) (1:100), and HLA-DP, DQ, DR (Dako, Denmark) (1:100), using the alkaline phosphatase anti-alkaline phosphatase method (Dako, Denmark). HLA-A2 expression was graded by a homogenous expression (>75%), a heterogenous expression (25–75%), or negative expression of the melanoma cells (Anastassiou *et al.*, 2003).

DNA extraction from paraffin-embedded tissue

SLN were divided into four sections. For DNA extraction in metastatic SLN, we extracted DNA from sections carrying melanoma cells, while in tumor-free SLNs DNA was extracted from central sections.

For DNA extraction from formalin-fixed, paraffin-embedded tissue, we used a modified version of the method described by Shibata (Shibata *et al.*, 1988; Graf *et al.*, 1996). Briefly, 15–20 tissue sections, each 10 μm thick, were cut, deparaffinized with xylene, and washed with 100% ethanol. The samples were digested for 2 days with digestion buffer and proteinase K at 37°C. DNA was extracted using phenol/chloroform, followed by sodium-acetate precipitation at −20°C overnight. After being washed with 70% ethanol, the DNA pellet was diluted in sterile water.

LCM and DNA extraction from captured cells

From 21 malignant melanoma specimens from 21 patients with a prominent lymphocytic infiltrate (Table 3), one 10-μm-thick tissue section was cut from the paraffin-embedded tissue block and stained using hematoxylin. Two lymphocytic infiltrates (approximately 500–2000 cells each) from various regions of the section, which were not adjacent to each other, were microdissected (Figure 1) with a PixCell II Laser Capture Microdissection System (Arcturus Engineering Inc., Mountain View, CA). The captured cells were immediately transferred to proteinase K-enriched digestion buffer (0.1 M Tris–HCl, 0.5 mM EDTA, 0.5% Tween 20). After an incubation period of 5–12 hours at 48°C, proteinase K was inactivated at 95°C for 10 minutes.

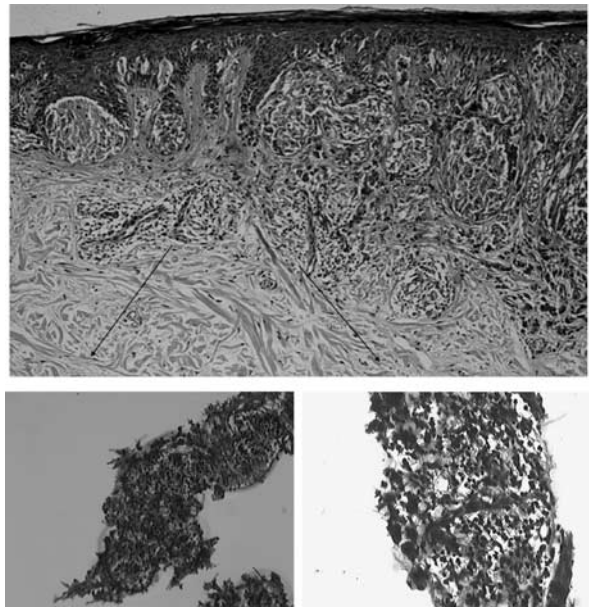

Figure 1. Histology of microdissected lymphocytes in primary malignant melanoma. Primary malignant melanoma with clustered TIL and dissected clusters of lymphocytes.

Presence of a sufficient amount of DNA was assured by amplifying a fragment of the β-globin gene (primers PCO3/KM38) as an internal control (Saiki *et al.*, 1985).

PCR assessment of the TCR γ gene

To theoretically cover all potential TCR γ-chain gene rearrangements, we used a parallel PCR approach with consensus primers (Schuhmann *et al.*, 1999), combining one of the four variable region family primers (Vγ1–8, Vγ9, Vγ10, and Vγ11) with a mixture of four joining primers (Jγ2, Jγp, Jγp1, and Jγp2). Gene segments Vγ1–Vγ8 were covered by the consensus primer Vγ2 (Graf *et al.*, 1996). Each DNA sample underwent four PCR reactions, one for each of the four variable region families of the TCR γ-chain gene. The T-cell line Jurkat was used as a positive control for the Vγ2 and Vγ11 primers. The T-cell line HSB2 was used for a positive control for Vγ9 and Vγ10 primers.

The PCR conditions for DNA extracted by conventional methods and for DNA isolated from LCM were identical, except for the size of the reaction. A 50-μl reaction with only 2-μl DNA input was used for analysis of DNA extracted from 15–20 10-μm-thick tissue sections. A 30-μl reaction was used with a DNA input of 3 μl for analysis of cells obtained by LCM. After initial heating at 94°C for 7 minutes, 45 cycles of PCR were carried out (94°C denaturation for 30 seconds, annealing 60°C for 30 seconds, and extension at 72°C for 30 seconds), followed by a 7-minute extension at 72°C.

The amplified products were run on 6% polyacrylamide gels, stained with ethidium bromide, and visualized under ultraviolet light. Contamination was avoided by strict technical methods. The unmounted hematoxylin-stained 10 μm sides were prepared using fresh and clean reagents. DNA extraction, PCR set-up steps, and the processing of PCR products were performed in different rooms. New gloves were used for each sample. Additionally, in all experiments, negative controls were carried out through all steps with test samples.

Sequencing

The two pairs of primary malignant melanoma and SLN, which showed a TCR γ chain gene rearrangement of the same V region, were subjected to DNA sequencing to determine whether the two clones were identical.

Four separate PCR reactions using the V primer with each J primer (Jγ2, Jγp, Jγp1, and Jγp2) were set up to explore which joining primer is used. An aliquot of the PCR products was run on a 6% polyacrylamide gel. The PCR reaction showing a monoclonal band on the gel was subjected to direct sequencing using the ABI Prism Dye Terminator cycle sequencing ready reaction kit (Applied Biosystems) according to the manufacturer's guidelines, using an ABI 373A DNA sequencer (Applied Biosystems).

CONFLICT OF INTEREST

The authors state no conflict of interest.

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